COMPARISON OF HYDROLYSIS EFFICIENCIES OF

MULTIPLE BETA-GLUCURONIDASE ENZYMES

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Abstract: In urine drug testing for pain management, dilute and shoot is a common method used to analyze drugs in urine. Beta-glucuronidases are enzymes that are used to cleave glucuronide conjugates off the drug metabolite, converting it back into the parent drug, allowing detection of the free drug. There are several different sources of beta-glucuronidases on the market today, including abalone, red abalone, limpet, and recombinant organisms. This research takes these different sources of beta-glucuronidase enzymes from different companies and determines the enzyme that have better hydrolysis efficiency. Hydrolysis efficiency is the conversion of total drug to the free drug by cleaving off the glucuronide portion of the drug. Six different sources were used along with six different drug-glucuronide conjugates, including opioids, benzodiazepines, and cannabinoids. Specimens were prepared using dilute and shoot methods, and guidelines were established to ascertain the most efficient and affordable. The guidelines stated that there could not be less than 40% conversion of the glucuronide conjugates to the target drug, it must work well across multiple drug classes and be cost efficient for a laboratory. Once the results were analyzed using these guidelines, a recombinant enzyme was considered to be the top enzyme of the six sources that were used in this research.

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ABBREVIATIONS

µL	microliters
mL	milliliters
M	molar
mM	millimolar
LC	liquid chromatography
GC	gas chromatography
LC/MS	liquid chromatography-mass spectrometry
LC/MS/MS	liquid chromatography-tandem mass spectrometry
°C	degrees Celsius
min	minutes
psi	pounds per square inch
rpm	revolutions per minute

CHAPTER I

INTRODUCTION

Drug testing is a significant part of most clinical laboratories. Accurate drug testing allows laboratories to test for and show precise concentrations of parent drugs that may be in an individual's system. Since some drugs may be present as metabolites that are conjugated to sugars known as glucuronides, beta-glucuronidase enzymes are frequently utilized for accurate drug testing. The enzyme assists in determining how much of a parent drug is present in an individual's system by converting the metabolite back to the parent compound. There are various sources of beta-glucuronidase enzymes, including *patella vulgata, red abalone, abalone and recombinant enzyme systems.*[5, 11, 14] Research across different drug classes such as benzodiazepines, cannabinoids, and opioids are currently underway to determine which type of beta-glucuronidase enzyme is most efficient at converting drugs to their free or parent forms for analytical purposes. Once a drug is converted to its parent form, it can then be detected by scientific instrumentation. For the instrumentation in this study, the drugs need to be unconjugated from the glucuronide portion to allow detection of the target drug rather than the glucuronide metabolite. This research is an attempt to demonstrate the most efficient and affordable beta-glucuronidase enzyme for conversion of metabolite to parent drugs, in order to further assist clinical laboratories in obtaining accurate concentration levels of drugs in an individual specimen.

In order for accurate detection via liquid chromatography tandem mass spectrometry multiple reaction monitoring, the target drugs need to be in their free or parent form. Laboratories depend on the ability of the instrument to detect the drugs accurately, so they can report the result with confidence. This research is significant because it may enhance sensitivity to certain drugs if an enzyme hydrolyzes them more efficiently. This research also considers the financial costs of enzymes by looking at how many samples can be analyzed using one bottle of the company's enzyme, as well as the personnel time it takes to convert a drug from its conjugated to its free form. Does the cost of the bottle and amount of samples that can be run using just that one bottle fit into a laboratory's budget? Additionally, this research looks at how the enzyme works across multiple drug classes with multiple drugs in each class, whereas most research on this topic focuses on one individual class at a time.

Multiple companies have manufactured beta-glucuronidase enzymes and tested them against those of competitors. Some companies provided optimal parameters such as incubation time, pH and volume of buffers, incubation temperature, and amount of beta-glucuronidase enzyme required. Other companies' optimal conditions were determined based on other research done using the enzyme or graphs from the manufacturer's website. The individual studies that these companies have produced, documenting the validity of their enzyme to produce the optimum results, are highlighted in the literature review, which also goes into detail on the glucuronidation process of a drug when it enters the body. In this research, beta-glucuronidase enzymes from different sources (*recombinant, limpet,* etc.) and six different companies were evaluated.

This research was executed by using a common sample preparation method found in drug-testing laboratories, known as the dilute and shoot method. The factors of the dilute and shoot method related to enzyme are the incubation time, pH of buffer, incubation temperature, and the amount of enzyme that is added to each sample. This research will be beneficial to other laboratories by providing information on which beta-glucuronidase enzyme converts drugs to their free form in the most efficient and cost-effective manner.

The aim of this paper is to determine which of the beta-glucuronidase enzymes performs best. In order to make this determination, the beta-glucuronidase enzyme performance was assessed by a set of developed guidelines. The enzyme must have a hydrolysis efficiency greater than 40%, which is defined as the percentage of the drug that is converted from its conjugated to its free form in the study. The enzyme must also perform well across different drug classes to be considered efficient. If the enzyme performs well in one or two drugs classes, but poorly in others, it was not considered efficient. Finally, laboratories will expect the enzyme to be cost-efficient. The purpose of this study is to find a beta-glucuronidase enzyme that will perform well in overall hydrolysis efficiency across multiple classes of drugs that is also affordable for laboratories.

CHAPTER II

LITERATURE REVIEW

Generally, drug testing is performed in clinical and forensic laboratories to determine the concentration levels of any legal or illegal drugs within a person's system. The first step in a drug test is to collect a specimen, which is typically a blood or urine sample but more commonly urine since it has a wider detection window than blood. Currently, the dilute and shoot method is utilized by most testing laboratories for urine specimen analysis. In dilute and shoot, a hydrolysis solution, which includes a beta-glucuronidase enzyme, buffer, and internal standard, is added to the sample and incubated at a specific temperature and for a specific amount of time. Afterwards, the sample is diluted down with sample diluent to reduce interference. The sample is then centrifuged and the supernatant is transferred to an amber vial for analysis on the instrument. The part of the dilute and shoot process that is critical to current research is the hydrolysis solution. In the hydrolysis solution, there are three components: the beta-glucuronidase enzyme, internal standard, and acetate buffer. Of those three components, this research will focus on the beta-glucuronidase enzyme.

When a drug, such as morphine, is taken by an individual, it enters the body in the parent, non-metabolized, form. However, upon absorption, the body metabolizes morphine and conjugates it to a glucuronide to form a compound called morphine-3-

glucuronide. In order to remove the glucuronide portion, a process called enzymatic hydrolysis typically using a beta-glucuronidase enzyme must occur. The beta-glucuronidase enzyme removes the glucuronide from the drug metabolite and converts it back to the parent drug. The process of glucuronidation increases the drug's water solubility, so the drug will be able to be effectively excreted from the body in the urine.[1]

The current research looked at six different glucuronidase enzymes from six different companies to determine which one efficiently cleaved off the most glucuronide tails across different drug classes including benzodiazepines, cannabinoids, and opiates. This study will identify which beta-glucuronidase enzyme cleaves off the most glucuronide portions more efficiently, which will allow for maximum drug recovery and detection in the urine sample.

<u>Pharmacokinetics (ADME)</u>

When a drug is taken, it must be absorbed, distributed, metabolized and excreted from the body. This process is captured in the acronym ADME which stands for absorption, distribution, metabolism, and excretion, respectively. Taken as a whole, this is referred to as pharmcokinetics, and describes what the body does to the drug. The effects of the drug on the body are part of pharmcodynamics, but that is less critical for this particular research.

Absorption

Absorption is the process by which drugs enter the bloodstream in order to be distributed to tissues where it might have an effect. There are multiple ways in which a drug can enter the system: oral, inhalation, intravenous, intramuscular, rectal, oral mucosa, intrathecal, dermal, ocular, or intranasal. The most common route of absorption is oral.

When absorbed orally, the drug goes through the gastrointestinal tract. There are some drugs that when they are absorbed from the gastrointestinal tract they immediately go to the liver through the hepatic portal vein. The drug will be metabolized by enzymes inside the liver to an extent that most of the active ingredient in the drug will not exit the liver and so therefore will not enter the systemic circulation. When a drug does not enter the systemic circulation, it leads to a decrease in the metabolism of the drug that can result in a substantial increase in peak plasma concentration and systemic availability. When a drug is absorbed by the gastrointestinal tract, one route it can take is to enter the portal circulation where it goes to the liver and then travels back to the gastrointestinal tract. This is called enterohepatic circulation. When a drug goes through enterohepatic circulation, the pharmacological effects of the drug and its metabolites are drawn out. If an individual wants the entire amount of drug to be absorbed, then the individual would absorb the drug intravenously. By absorbing a drug intravenously, the individual is getting the entire drug in their system and getting the drug into the bloodstream faster. With the other routes of absorption, only a variable amount of the drug is getting into the system. The bioavailability, which is the amount of drug absorbed relative to the amount that is administered, can be affected by different properties. Those properties include solubility, concentration, surface area, blood supply, and pH. Each one of these properties places a role in how much of the drug that is administered actually gets absorbed into the body. The solubility of a drug plays an important role because it the drug is in a tablet form; it must be broken down first before being taken into the bloodstream. If a drug tablet is coated or sustained release, then the drug will absorb more slowly than other forms of tablets. Drugs that are in an aqueous medium such as immediate release tablets, will absorb more rapidly.

The more concentrated a drug is, the faster it will absorb. The small intestine and the stomach both have large surface areas which help the process of absorption. Increased blood supply also helps the process the of absorption. By increasing the blood flow, the absorption rate of the drug will increase as well. Finally, pH will affect the absorption rate. In order for the drug to enter the bloodstream, it must pass through membranes. Depending on whether or not the drug is acidic or basic will determine how much of the drug will stay ionized or unionized in either the stomach or small intestine. If a drug becomes more ionized, then the amount of drug that is absorbed will be less than that of a unionized drug. In order to determine how much of a drug will be non-ionized, the Henderson-Hasselbach equation is used.[26]

Distribution

Distribution is the transfer of the drugs from one part of the body to another. The highly perfused tissues such as heart, liver, kidney, and brain will get most of the absorbed drug. Tissues such as muscles and fats take more time to reach equilibrium with the blood so those tissues don't get as much of the absorbed drug which makes those tissues less-perfused. Factors that affect the absorption of the drug also affect the distribution. Drugs that are more lipid soluble will cross the membranes more easily, which means more drug present in the blood which means more drug will be distributed throughout the body. The pH also has an effect on the distribution because it is the unionized part of the drug that is able to move across the membrane and be distributed into the blood stream. Plasma protein binding plays a role in distribution in that only unbound or free drugs can leave the blood and be distributed into the tissues. Those drugs that are bound to proteins will stay in the blood longer because of their size. Therefore, the drugs that are bound to proteins won't be able to reach their intended site and produce the effects that was intended. If multiple highly bound protein drugs are taken, an interaction can occur that could lead to an increased pharmacologic activity or toxicity. One important concept that has come from distribution is the volume of distribution. The volume of distribution is the amount of fluid that a drug dose would have been distributed if the total dose had remained in the blood.[26]

Metabolism

Metabolism is the process in which the drug is modified for elimination from the body. This process occurs with the help of enzymes or catalysts. Metabolism usually occurs in the liver. There are two different parts of metabolism: Phase I and Phase II. Phase I metabolism includes the reactions that convert a parent drug to a more polar drug by inserting a polar functional group. Some examples of Phase I reactions are reduction, N-dealklyation, ester hydrolysis, and aromatic hydroxylation. Phase I reactions form more polar metabolites that can be either pharmacologically active or inactive. Then, in Phase II reactions, the drug or metabolite from Phase I is chemically changed into a compound that is soluble enough to be excreted out of the body.[26] Phase II metabolism conjugates a drug which produces a drug that has a higher polarity, greater water solubility, and easier to eliminate from the body. Most Phase II metabolism reactions are catalyzed by transferase enzymes. The Phase II metabolism reaction product that is formed is usually the inactive metabolite, compared to Phase I where the active metabolite is formed. There are four major conjugation reactions in Phase II metabolism: methylation, sulphation, acetylation and glucuronidation. The majority of Phase II reactions occur in the smooth endoplasmic reticulum of liver cells. Liver is the main site of metabolism because that is where the highest concentration of metabolizing enzymes is located.[27] Phase I metabolism is usually followed by Phase II metabolism, although the structure of the drug determines whether or not a drug will undergo Phase I metabolism or not.[26] A drug does not have to go through a Phase I metabolism reaction to be able to go through a Phase II metabolism reaction.

An important concept with metabolism that was mentioned in the previous section of absorption is the first pass effect or first pass metabolism. In the first pass effect, when a drug is administered orally and is absorbed by the gastrointestinal tract, the drug will be transported through the portal vein straight to the liver, where it is metabolized. When a drug undergoes first pass metabolism, a small portion of the active drug will reach the systemic circulation in order for it to reach its target tissue. For drugs that have a high first pass effect, users will not take a drug orally instead they will administer the drug through other routes such as intravenously, sublingually, or buccally so the user can get the desired effect of the drug.[28]

Glucuronidation

Glucuronidation is a vital part of drug metabolism. Glucuronidation is a type of conjugation reaction. Conjugation reactions with hydrophilic compounds result in a large increase of xenobiotic hydrophilicity, enabling the xenobiotic to be efficiently excreted from the body. Even though most xenobiotic compounds are highly water soluble, they are unable to be diffused across the plasma membrane. Therefore, the transport of the xenobiotic compounds involves the transport across the canalicular membrane into bile or across the sinusoidal membrane into blood. The reason for the xenobiotic compound to be unable to readily pass through the plasma membrane is because the metabolites formed by conjugation reactions, such as glucuronides, have lower log P and a much greater total polar surface area (TPSA) than their parent drug. Because of these parameters, the metabolite is unable to diffuse passively across biological membranes. The drug conjugates are actively transported into the blood by MRP1 and MRP3 is used to transport on the sinusoidal membrane.[2]

For mammalian species except the cat family, glucuronidation is a major pathway for transforming a xenobiotic so that it can be excreted from the system.[2] Glucuronidation is considered to be a phase II reaction; however, glucuronidation is considered to be the initial biotransformation step in the metabolism of many drugs.[3] The primary helper in glucuronidation is the co-factor UDP-glucuronic acid (UDPGA). UDP-glucuose, UDP-xylose, and UDP-galactose can also be used; but for this research, UDP-glucuronic acid is the one that will be used the most with the drugs that are being researched. The enzyme that is used to catalyzed the reaction is UDP-glucuronosyltransferases (UGTs).[2] UGTs are membrane-bound enzymes that transfer the glucuronic acid from the UDPGA to a nucleophilic functional group such as phenols, carboxylic acid, amines, sulfhydryl groups, and nucleophilic carbons. The addition of this glucuronic acid to certain molecules increases the polarity and the water solubility. By adding the glucuronic acid, it increases the ability to be excreted into bile, urine, or both.[4] The UGTs catalyze the reaction which takes place predominantly in the endoplasmic reticulum of the liver and other tissues such as the kidney, GI tract, lungs, and other organs.[2]

The site on the xenobiotic where the glucuronidation occurs is usually an electron-rich O, N, or S heteroatom. The glucuronidation reactions that involve UGTs are S_N2 substitution reactions. In those substitution reactions, the nucleophilic heteroatom of the substrate attacks the C_1 atom of the UDPGA, which results in a xenobiotic glucuronide in the β-configuration. The substrates used for glucuronidation are those that are small lipophilic compounds that contain certain functional groups which form different glucuronides. Aliphatic alcohols and phenols form the *O*-glucuronide acetals. Acyl glucuronides are formed from carboxylic acids. *N*-glucuronides are formed from aromatic and aliphatic amines. Free sulfhydryl groups form the *S*-glucuronides. With *N*-glucuronidation, there are substrates with tertiary amines that when they undergo N-glucuronidation reaction, they will form positively charged quaternary glucuronides. Some of these positively charged quaternary glucuronides can be carcinogenic.[2]

The co-factor for this reaction, UDPGA, is synthesized from glucose-1-phosphate. The linkage between the glucuronic acid and UDP is an α-configuration. This particular configuration protects the co-factor from hydrolysis by β-glucuronidase. One problem with that is the glucuronides of drugs have a β-configuration. The inversion of this configuration occurs because glucuronides are formed by nucleophilic attack by an electron-rich atom on UDPGA. The nucleophilic attack occurs on the opposite side of the linkage between glucuronic acid and UDP, which as mentioned above, is an α-configuration.

Drugs that are conjugated with glucuronides are substrates for β-glucuronidase. The β-glucuronidase activity is mainly present in the intestinal microflora. They can also be present in the lysosomes of some mammalian tissues. The intestinal enzyme can release the aglycone, which can be reabsorbed and then enter a cycle called enterohepatic circulation. The aglycone is the parent compound or its unconjugated metabolites. Once the aglycone enters the enterohepatic circulation, it can delay the elimination of the drugs. *N*-glucuronides are generally hydrolyzed by β-glucuronidase rather slowly. Some *N*-glucuronides are resistant to hydrolysis by β-glucuronidase. Some *N*-glucuronides are hydrolyzed in acidic conditions (i.e. urine), which can give the impression that the parent compound was eliminated unchanged. Determining whether the glucuronide will be excreted via the bile or the urine depends on the size of the aglycone. In a study done on rats, if the molecular weight of the aglycone was less than 250, it was excreted in the urine. Aglycones that were higher than 350 were excreted in the bile.[2]

The carboxylic acid part of the glucuronic acid promotes the excretion of the xenobiotic because it increases the aqueous solubility and TPSA of the xenobiotic as well as it is recognized by the biliary and renal organic anion transport systems. When it is recognized by these systems, it allows the glucuronide to be excreted into urine and bile.[2]

Excretion

Excretion is the process of removing the drug from the body. The two main routes in which the drug is excreted from the body is through the kidney (renal) or the liver (hepatic). The liver is the major site of drug metabolism. Once the drug is cleared from the liver, it is stored in bile from the liver that is stored in the gall bladder. From there it enters the small intestines where the drug is then eliminated in the feces. There are a couple of factors that affect the clearance of the drug from the blood to the liver. One factor is the blood flow to the liver. Certain diseases can either decrease or increase the blood flow to the liver. Another factor is the ability of the liver to remove the drug from the blood. Renal excretion is a function of filtration, secretion, and reabsorption. Urine is a major excretory pathway for drugs. Urine drug testing is what most clinical laboratories use to test for certain drugs. The drug can also be excreted through the breath. One good example of a drug being excreted through the breath is alcohol. Another way a drug can be excreted is through sweat.[26]

Sources of Enzymes

There are different sources of beta-glucuronidases that are used in laboratories. Some of the different sources include abalone, red abalone, limpet, and recombinant organisms. While there may be other sources available, these particular sources are the ones that were used in this research project.

Abalone is part of the phylum *Mollusca.* The *Mollusca* group includes the clams, scallops, sea slugs, octopuses and squid. The abalone has a soft body that is surrounded by a mantle, anterior head and a large muscular foot.[19] Most abalone have been harvested in Southern California. In the late 1800s, Asians began harvesting abalone intertidally which was in between successive tides. Then in the early 1900s, they began harvesting the abalone by diving. As a result of this kind of harvesting, the abalone population began to decline very quickly. Fast forward 50-60 years, with the developments of scuba diving, scuba divers could now dive more than 100 feet. These developments led to the thousands of abalone being taken from the ocean. In 1996, almost less than 10% of what was usually caught by scuba divers could actually be harvested by commercial abalone divers. Not only was the catching of abalone leading to the decrease, but also the environment. Several El Ninos that came in the 1908s and 1990s brought warmer water which lead to the decreased nutrient levels and decreased the kelp levels which is a main food source for abalone. Due to the decreased population of the abalone, the California State Department of Fish and Game banned the taking of abalone by either sport or commercial diving due to the fear that the species would become extinct. In 1970, Dr. Daniel Morse began to research how to spawn abalone. He found that he could "fool" the abalone into sensing hydrogen peroxide in the water and mistaking it for their own. The abalone would in turn release a hormone and the abalone would spawn. This research led to the increase in mariculture farms that would spawn abalone in the 1970s. It wasn't until the 1980s that the farms actually started producing the abalone. Most of the farms are private enterprises. Few of the farms provide maricultured abalone to restaurants in Southern California.[20]

Red abalone is one of the largest species of abalone in the world. They are well-suited for farming both in land-and-ocean based operations. Most of the red abalone live in the intertidal and sub-tidal zone.[21] Red abalone belongs to the same phylum as abalone *Mollusca.* They are most commonly found in Northern California. They are slow-growing herbivores who feed off of drift kelp. Like abalone, the red abalone population has decreased due to overharvesting, predation by sea otters, and disease. Fishing for abalone peaked in the 1950s and 60s which lead to a decline in the populations. Because of this, the ban of commercial fishing was done in 1997. As of today, red abalone is legally harvested on a restricted basis only in Northern California. Abalone Recovery and Management Plan was formed in 2005 by the Fish and Game Commission to regulate the recreational fishery in Northern California. It was also set up to aid in the recovery of the depleted abalone in the rest of California. As with abalone, red abalone farming has become a popular way of harvesting the red abalone.[22]

Limpet is an herbivorous marine snail that lives along the coast of Western Europe. Limpets can be found in the intertidal zone which makes them well-adapted to an amphibious life. Limpets are part of the same phylum as the abalones, the *Mollusca.* They have a thick, conical shell and strong muscular foot. With these type of structure, it gives the limpet a defense against predators in and out of the water.[23] Limpets feed on the algae which is found on the rock on which they live. Limpet farming is not as common as with the abalone.

Recombinant is another source of enzyme but recombinant is different from the other three sources already mentioned. Recombinant is primarily made in the laboratory by using a transformation process. With the transformation process, a piece of DNA is inserted into a vector, then that particular piece of DNA is cut with a restriction enzyme. Using DNA ligase, the DNA insert is ligated into the vector. The vector is inserted into a host cell where the host cell produces the enzyme. In this case, the host organism would be E. Coli. Different vectors have different properties to make them suitable to different applications, but this is the mechanism of recombinant glucuronidase production that produced the enzyme used in this research.[29]

Drug Classes

Three different drugs classes were used throughout this research: benzodiazepines, opioids and cannabinoids. Benzodiazepines are used mainly for anxiety and other conditions. Benzodiazepines work in the brain by binding to a receptor and affecting the neurotransmitter releasing nerves that communicate with other nerves in the body. One of the neurotransmitters that are affected is the gamma-aminobutyric acid (GABA), which is an inhibitory neurotransmitter that suppresses the activity of nerves. Some examples of benzodiazepines include oxazepam, lorazepam, temazepam, alprazolam, diazepam, and flurazepam. Benzodiazepines are a Schedule IV drug which means they have a low potential of abuse and low risk of dependence.

Opioids are a drug class that has gained a lot of attention over the years and are used as analgesics, or pain relievers. It is a drug class that is mainly used in pain management, as they act on the opioid receptors to produce morphine-like effects. Some examples of opioids include fentanyl, hydrocodone, oxycodone, morphine, codeine, and buprenorphine. Most of the opioids are under the Schedule II drug class which are drugs that have a high potential for abuse, with use potentially leading to severe psychological or physical dependence such as hydrocodone and oxycodone. There are some opioids that are in the Schedule III class which has a moderate to low potential for physical and psychological dependence, such as buprenorphine.

Cannabinoids are chemical compounds that act on the cannabinoid receptors in the body to alter the release of neurotransmitters in the brain. Cannabinoids are any various chemical constituent of cannabis or marijuana. As of today, cannabinoids are currently a Schedule I drug which means that there are no currently accepted medical use and there is a high potential for abuse. However, some people do self-treat themselves with this drug. Currently, there are four states (Colorado, Washington, Oregon and Alaska) plus District of Columbia that have passed laws for legalized cannabis for recreational use. Twenty-three states plus District of Columbia have passed laws that cannabis can be used for medicinal purposes.[30]

Drug Testing Method

In most urine drug testing laboratories, dilute and shoot is the method which is most commonly used. In dilute and shoot, a technician is diluting the drugs in the urine sample and then "shooting" it onto the instrument. Within a dilute and shoot method, there are different steps that must be taken. When a urine sample comes into the laboratory, the sample is transferred from the original container to a labeled tube. A hydrolysis solution must then be made. The hydrolysis solution contains buffer, internal standard and a beta-glucuronidase enzyme. The buffer is used to help minimize the change in pH when base or acid is added to the solution. Buffers are needed for the enzyme stability so if the pH changes during the method, it won't affect the enzyme in a negative way. In our method, acetate buffer is used because it has a pH in which the beta-glucuronidase enzyme will work. If the pH is at a higher or lower pH, the enzyme will be unable to work the way it is supposed to. A consistent amount of internal standard is added to each sample so that we can have something to compare our unknown samples with during data analysis. In the internal standard solution, deuterated compounds are used as our standards. Deuterated drugs are drugs in which the hydrogen atoms are replaced by a heavier stable isotope deuterium. Deuterated internal standards are used because deuterated standards are inexpensive and are easier to incorporate into a method. Finally, a beta-glucuronidase enzyme is added, which will help cleave off the glucuronide portion from conjugated drugs. Once the hydrolysis solution is made up, an aliquot is added to each sample. The samples are then incubated for a certain time and temperature. The amount of incubation time is dependent on the temperature preferred by the enzyme for maximal activity. If a sample is being heated at 35°C, the samples will need to be incubated longer than when a sample is being heated at 65°C. Once the samples are heated and incubated, sample diluent is added to dilute the sample down even further. After sample diluent is added, the samples have to be centrifuged. Centrifuging the samples is critical because we need to get rid of the solid particles in the sample. If solid particles are still present when the samples are injected on the instrument, the solid particles will clog up the column which would cause a pressure build up in the system, which can lead to failure through leaks. Once they are centrifuged, the supernatant sample is transferred into vials to be put on the instrument for analysis.

The use of a hydrolysis solution is not always necessary for a laboratory, as not all drug classes form glucuronides. Therefore, the use of a hydrolysis solution would be beneficial for the classes of drugs that form glucuronides. Also, if a method is looking for the glucuronides in urine, then hydrolysis wouldn't be necessary because the beta-glucuronidase would be getting rid of what the method is looking for. If the laboratory's method looks for the unconjugated drugs and it is a glucuronide forming class, then hydrolysis solution would be necessary.

Instrumentation

When performing a dilute and shoot extraction in urine, most clinical laboratories use liquid-chromatography with tandem-mass-spectrometry (LC-MS/MS).

In the liquid chromatograph (LC) part of the LC-MS/MS, there are multiple different parts which include: pumping system, sample-injection system, columns, and detectors. There are 5 requirements according to Skoog that a modern LC should have: (1) generation of pressures up to 6000 psi, (2) pulse-free output, (3) flow rates ranging from 0.1-10 mL/min, (4) flow reproducibilities of 0.5% relative or better, and (5) resistance to corrosion by a variety of solvents.[24] Unlike with gas chromatography (GC), high pressures are not an issue with the LC because liquids are not very compressible and so therefore do not pose an explosion risk. The risk that is involved is solvent leakage. Solvent leakage can cause a fire or environmental risk with other solvents. Special care should be taken to prevent a solvent leakage. In most commercial chromatographs, the pumping system is a reciprocating pump. A reciprocating pump is one in which a small chamber that has the solvent is pumped in a back and forth motion of a motor-driver piston. There are two ball-check valves that open and close alternatively. The valves control the flow of solvent into and out of the cylinder. The disadvantage of using this kind of pump is that it produces a pulsed flow which appear as a baseline noise on the chromatogram. Dual pump heads and elliptical cams are used to help alleviate the pulsed flow in the pumps. The advantages of using this pump is the small internal volume, ability to reach high pressures, adaptability to gradient elution, large solvent capacities, and the ability to have constant flow rates.[24]

Another part of the LC system is the sample-injection system. The sample-injection system is an important part because it is where the sample is being introduced into the instrument. The most common way that a sample is introduced onto the column is through a sampling loop. In a sampling loop, the sample is injecting into the loop while the loop is switched out of the LC flow path. Once the loop is filled with the sample, the loop is switched back to the LC flow path where it is then swept onto the head of the LC column.[25] Sampling loops can provide a range of sample sizes from 1 μL to 100 μL and also allowing pressures to raise to 7000 psi. In most chromatographs, an auto injector is installed. An auto-injector are capable of injecting samples in the LC from vials either on a tray or in a carousel. The auto injectors usually already have a sampling loop and a syringe pump that can hold a volume of 1 μL to 1 mL.

The next part of the LC system is the column. Columns can be made from various sources such as stainless-steel tubing, heavy-walled glass tubing, and polymer tubing.

There are multiple different packed columns that range according to their size and the packing that is used. Most columns range from 5-25 cm long. The inside diameter ranges from 3-5 mm. Particle size inside the column ranges from 3-5 µm. The column used in this study had a 2.7 mm diameter range and 2.7 µm particle size. The packing inside of the column usually consists of silica. In order to improve the life efficiency of the column, a guard column is added. The guard column removes the particulate matter and contaminants from the solvents. Once the guard column has become too contaminated, it is then replaced. By replacing the guard column, it helps improve the efficiency of the column itself. Guard column packing should be similar to the packing of the column being used. Reverse phase LC is the most common liquid chromatography method that is used in the LC instrument. Reverse phase HPLC uses a polar mobile phase and non-polar stationary phase, and the most polar compounds will elute off the column first.

The next part of the LC system is the detector. The most common detector that would be coupled with a LC is a mass spectrometer. Mass spectrometers aid in the identification of the compounds as they come off the column. When the compound comes off the column, the first part it encounters in the mass spectrometer is the ion source. The ion source is where the compound is formed into gaseous analyte ions. There are two different basic types of ion sources: gas phase and desorption. In the instrument that is used in this research, the desorption ion source is used. The advantage of using the desorption ion source is that volatile and thermally unstable samples can be analyzed. Within the desorption ion source type, there are other different types of ion sources. Electrospray ionization is what our instrument uses. In electrospray ionization, the ionization takes place under atmospheric pressures and temperatures. Part of the sample is pumped through a stainless steel capillary needle at a rate of microliters per minute. The needle is kept at several kilovolts. As a result, a charged spray of droplets is emitted and goes through the capillary. Within the capillary, evaporation of the solvent and attachment of the charge to the analyte occurs. When the droplet becomes smaller, the charge density becomes greater until a point called Rayleigh limit which is where the surface tension can no longer support the charge. When the limit is hit, a Coulombic explosion occurs and the droplet is broken apart into smaller droplets.

Once the analyte has been ionized, it now travels to the mass analyzer. In the tandem mass spectrometer, there are three quadrupoles, such that these instruments are referred to as triple-quadrupole spectrometer. In a quadrupole mass filter, a variable radio-frequency is also applied to each rod. When the ions are accelerated into the space between the rods by a potential difference of 5-10 V, a mass spectrum can be produced. While this is occurring, the ac and dc voltages on the rods are increasing simultaneously while maintaining their constant ratio. The ions that have the certain m/z charge that is being targeted will reach the signal transducer and produce signal. The other non-selected charges will strike the rods and become neutral molecules. With a tandem mass spectrometer, there are two different mass analyzers present. The first mass analyzer looks for a precursor ion. Once the precursor ion is chosen, it is sent to the collision cell, which is also a quadrupole. Inside the collision cell, the precursor ion will react with a collision gas and burst into fragments and produce product ions. The product ions will then travel to the second mass analyzer where it will look for another m/z ratio. Those particular ions will then travel to the detector where a spectrum will be produced.[24]

Past Research

Research was conducted using six different enzymes to determine which one of them had a better hydrolysis efficiency. Of the six different enzymes, three were abalone, one was red abalone, one was limpet, and one was recombinant or genetically modified in the lab. The six different enzymes that were used were from six different companies: Company A, Company B, Company C, Company D, Company E, and Company F.

In a study done by Malik-Wolf, researchers used the abalone β-glucuronidase; benzodiazepines, opioids, and synthetic cannabinoids were the drugs used to determine the hydrolysis efficiency. In this study, solid phase extraction (SPE) was the main technique used. Each drug class had different hydrolysis parameters. For the benzodiazepines, 20 µL of internal standard was added to 1 mL of specimen. Two mL of 0.1 M sodium acetate buffer was also added. The specimen was hydrolyzed with 50 µL of the β-glucuronidase followed by an incubation at 70°C for 30 min. After the incubation time, the samples were centrifuged for 10 min at 3000 rpm. The specimens were then run on the instrument according to the lab's instrument parameters.

For the opioids, 2 mL of the specimen was mixed with 100 µL of internal standard. The urine samples were then hydrolyzed with 1 mL of hydrogen chloride (HCl) in an autoclave at 15 psi for 45 min and allowed to cool afterwards. The specimens were then reacted with 20 µL of 10% hydroxylamine and incubated at 70°C for 15 min. The samples were centrifuged at 3000 rpm for 5 min before being put on the SPE column. Finally, the synthetic cannabinoids were extracted using a liquid-liquid extraction. Two mL of specimen was added with 50 µL of internal standard solution. This was followed by hydrolysis using 1 mL of 0.5 M phosphate buffer and 50 µL of β-glucuronidase. The specimens were incubated at 55°C for 30 min. Samples were cooled to room temperature where HCl and chlorobutane was added to the samples and they were centrifuged at 3000 rpm for 5 min. Organic layer was transferred to a clean vial where it was evaporated under nitrogen at 55°C. The extracts were reconstituted in 50 µL of mobile phase and then transferred to vials to be put on the instrument.

The results of this study showed that the abalone β-glucuronidase was able to efficiently hydrolyze the benzodiazepines within 20% of the expected concentrations. The opioids showed similar results to the other enzyme that was previously done. The abalone β-glucuronidase was able to completely hydrolyze the morphine-3-glucuronide. Researchers also found that the hydrolysis of the synthetic cannabinoids had shown increased results from the method that was currently in use.[6]

Using a recombinant enzyme, results seems to have shown good hydrolysis efficiency with buprenorphine and norbuprenophrine. In a study that was presented at the Society of Forensic Toxicologists in 2014, the recombinant enzyme had a hydrolysis efficiency of 103.8% and 92.6% for buprenorphine and norbuprenorphine respectively. The hydrolysis parameters for the drugs were 100 µL of urine, 20 µL of the enzyme, 38 µL of buffer and 342 µL of water. The study found that complete hydrolysis of norbuprenorphine was done at an incubation time of 30 min at 65°C. The maximum buprenorphine hydrolysis was found to be without heating or incubation time.[8]

In regards to the opioids, one study done showed that they could achieve complete hydrolysis of the opiate metabolites. The researchers used 30 µL of fortified urine that had been spiked with the various opioids. Varying amounts of the enzyme was used; anywhere from 5-80 µL was used. Depending on how much beta-glucuronidase was used determined the incubation time of the samples. The incubation temperature was fixed at 55°C. Both the buffer and internal standard were fixed at 90 µL and 30 µL respectively. The samples were run using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results showed that for the urine samples that were incubated for only 15 minutes, and contained 60 µL of beta-glucuronidase, the enzyme recovered 100% of morphine, 94% of oxymorphone, 98% of hydromorphone, and 84% of codeine. Samples incubated at 30 minutes showed a 90% hydrolysis efficiency for all four glucuronides using only 30 µL of enzyme. Researchers concluded that recombinant enzyme was effective in achieving complete hydrolysis of opiate metabolites within one hour using 60 µL or less of the enzyme.[9]

In a study done by Morris, researchers compared the effectiveness of two different enzymes: β-glucuronidase from abalone and recombinant enzyme. Drug-free urine was fortified with glucuronides of oxazepam, lorazepam, and temazepam at 2,500 ng/mL. An internal standard mix was made with the concentration of each internal standard at 1,000 ng/mL except 7-aminoclonazepam which was at 5,000 ng/mL. The hydrolysis efficiency was assessed in triplicate. Two incubation temperatures were used: 55°C and room temperature (RT). For the 55°C temperature, the incubation times were 5, 15, 30, and 60 minutes. For RT, the incubation times were 0, 5, 10, and 15 minutes. Once hydrolysis was complete, the samples were centrifuged and injected onto the instrument. The maximum hydrolysis of the glucuronides was found at an incubation time of 45 minutes at 55°C and at 5 minutes at RT. Researchers stated that there would be a variation in enzyme hydrolysis efficiency due to the influence of the chemical structure and its reactivity with the enzyme. Overall, the study showed that recombinant β-glucuronidase was a cleaner extract. The study also showed that by using that enzyme, researchers can decrease processing time due to no incubation time.[10] Eliminating the incubation time step would help speed up the process of getting results faster.

In one study, enzyme from red abalone was used to find whether or not it could hydrolyze cannabinoids. The parameters that were found to have the maximum hydrolysis efficiency was using 2000 units of enzyme, 2M sodium phosphate buffer at pH of 6.8, incubation time at 16 hours for 37°C. The enzyme was able to have the highest hydrolysis efficiency for the 11-Nor-9-Δ^9-tetrahydrocannabinol (THCCOOH) compound with efficiencies over 99%. The researchers are going to take these results and use them during clinical studies that will be investigating monitoring minor cannabinoids to determine if researchers can distinguish frequent users from occasional abusers.[12]

Another study used enzyme from red abalone to look at the hydrolysis efficiency of morphine and codeine. The urine specimens were spiked with codeine-6-glucuronide, morphine-6-glucuornide and morphine-3-glucuronide. Internal standard was added as well as a buffer solution at pH 5. The specimens were incubated at 60°C and 68°C for 30 and 60 minutes. For both codeine and morphine, the greatest hydrolysis efficiency came when the incubation time was 60 min at 68°C. The mean recovery was at 95.8%. They also compared this enzyme hydrolysis to using acid hydrolysis and found that both hydrolysis methods produced similar results. The researchers concluded that enzyme from red abalone is capable of achieving complete hydrolysis of both codeine and morphine from their glucuronides after only 60 minutes of incubation time.[13]

Enzyme from limpet helps eliminate the conversion of 6-monoacetylmorphine (6MAM) to morphine.[14] One study that was done to compare this particular enzyme to other types of enzymes such as the E. Coli enzyme and the native limpet enzyme. The study also looked at how much 6MAM was converted over to morphine. The parameters for this study included enzyme concentration at 65,000 units/mL at pH 5.2 for the limpet enzyme. Equal volumes of sample and enzyme stock were added, and incubated at 60°C. For the limpet enzyme, the study found that codeine glucuronide conversion was above 95% after 2 hours of incubation time. They also found that the conversion of 6MAM to morphine was below 1% after 2 hours.[15]

There was a study done that used enzyme from abalone that determined the hydrolysis efficiency of codeine and morphine. The study used 1 mL of urine sample that contained all the internal standards. One mL of 100 mM acetate buffer at pH 5 was added along with 50 μL of the enzyme. Samples were vortexed and incubated at 65°C for 1 hour. The results showed that for the morphine-3-glucuronide, the hydrolysis efficiency was above 90%. However, for morphine-6-glucuronide and codeine glucuronide, the hydrolysis efficiency was not all that efficient. Overall, the enzyme from abalone gave the best efficiency out of the three enzymes that were used in the study.[17]

CHAPTER III

METHODOLOGY

In this research, the comparison of different beta-glucuronidases is explored. The purpose of this research is to determine which of the beta-glucuronidases will produce the best hydrolysis efficiency across different drug classes. The hydrolysis efficiency is determined by three different factors: incubation time, incubation temperature, and the amount of enzyme added to the sample. This section explains the procedures that are performed in this research. It outlines materials needed, solution preparation, extraction procedure, and instrumentation needed for this research. Since this research did not deal with human subjects or protected health information (PHI), no institutional review board (IRB) approval was needed.

Materials

All samples were analyzed using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a 2 LC-AD solvent pumps, an SIL-20A HT autosampler, a CTO-20A column oven, a CBM-20A control module, and an FCV-20AH2 diverter valve. The HPLC system was attached to a Shimadzu LCMS-8040 liquid chromatography tandem-mass spectrometer (LC-MS/MS) system. A Restek® Raptor™

Biphenyl guard column (2.7 um, 5 x 3.0 mm) and column (2.7 um, 50 x 2.1 mm) were used for LC separation (Restek, Bellefonte, PA). The LC-MS/MS is used for clinical work in the pain management laboratory located on Oklahoma State University Center for Health Sciences (OSU-CHS) campus in Tulsa, OK.

Reference standards and glucuronides were purchased through Cerilliant (Cerilliant Corporation, Round Rock, TX). Blank drug-free urine was purchased through UTAK (UTAK Laboratories, Inc., Valencia, CA). Enzymes were acquired through different vendors. The enzymes were mostly in liquid form at 100,000 activity units. Company E's enzyme was purchased in a powder form and had to be reconstituted in acetate buffer to get into liquid form.

The drugs that were of interest to this research are morphine-3-β-D-glucuronide, codeine-6-β-D-glucuronide, lorazepam glucuronide, oxazepam glucuronide, (+)-11-Nor-Δ9-THC-9-carboxylic acid glucuronide, and buprenorphine-3-β-D-glucuronide. The structures of the drugs are found in Figure 1.

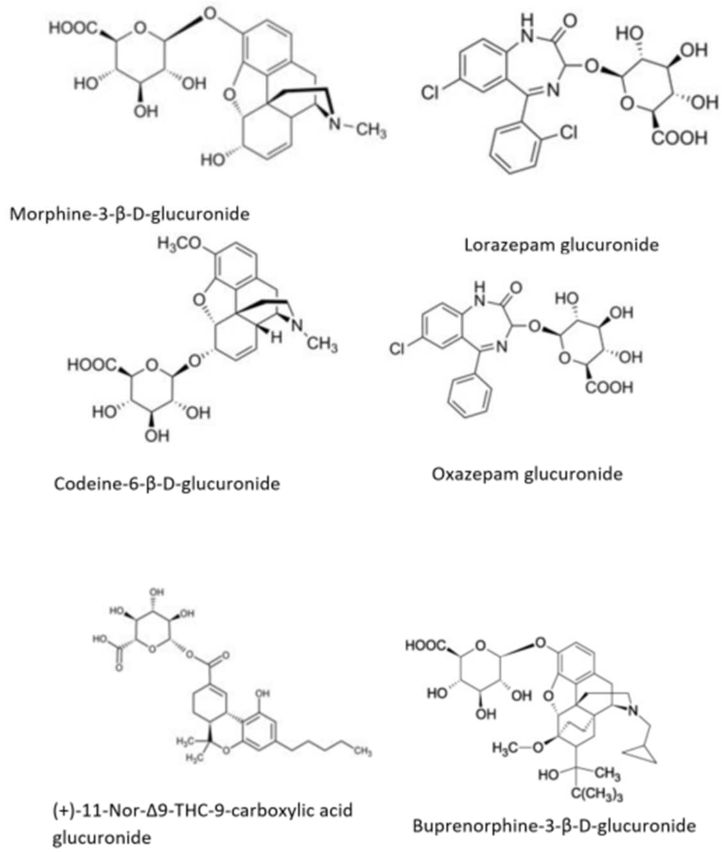

Figure 1: Structures of the 6 glucuronides that were used during the research

Solutions

Three different master mix solutions were prepared: a calibrator solution, glucuronide solution, and the Internal Standard (IS) solution using deuterated drugs.

Two different curves were used during the research. The first curve was made using seven different calibration points. Each calibration point was a multiple of the lower limit of quantitation (LLOQ). The five calibration points were 0.5c, 1c, 2.5c, 7.5c, 10c, 15c, and 25c. The 25c is the upper limit of quantitation (ULOQ) for the method and is the highest concentration that can be accurately quantitated. The concentration for each point is listed in Table 1 below.

Table 1. Concentrations for each calibrator point. Concentrations of each analyte at the respective calibrator point

ANALYTE	0.5C	1C (LLOQ)	2.5C	7.5C	10C	15C	25C (ULOQ)
BUPRENORPHINE	10	20	50	150	200	300	500
CODEINE	10	20	50	150	200	300	500
LORAZEPAM	10	20	50	150	200	300	500
MORPHINE	10	20	50	150	200	300	500
OXAZEPAM	10	20	50	150	200	300	500
THCA	10	20	50	150	200	300	500

Serial dilutions were done to make working solutions for each calibrator point. The serial dilution is located in Table 2 below.

Table 2. Serial Dilution Table. Serial dilution table for the calibrator points for the first curve that was used.

SAMPLE NAME	CALIBRATOR SOLUTION	25C	15C	10C	7.5C	2.5C	1C	POOLED URINE	TOTAL VOLUME
25C	87.5							787.5	875
15C		516						344	860
10C			510					255	765
7.5C				405				135	540
2.5C					190			380	570
1C						210		315	525
0.5C							175	175	350

The serial dilutions were done using blank, drug-free urine. Fifty µL of the working solutions were aliquoted out into separate labeled Eppendorf tubes and then placed in the freezer until needed.

The second curve that was used was from the Oklahoma State University Clinical Laboratory Services (OSU-CLS). These curves were pre-made for use in urine drug testing laboratory. The OSU-CLS uses a five-point calibration curve. The concentrations for each calibrator point are found in Table 3 below.

Table 3. Concentrations for each calibrator point on FTTL curve. Concentrations of each analyte at the respective calibrator point.

ANALYTE	1C (LLOQ)	2.5C	7.5C	15C	25C (ULOQ)
BUPRENORPHINE	25	62.5	187.5	375	625
CODEINE	20	50	150	300	500
LORAZEPAM	20	50	150	300	500
MORPHINE	20	50	150	300	500
OXAZEPAM	20	50	150	300	500
THCA	150	375	1125	2250	3750

Glucuronide Preparation

Stock concentrations of each glucuronide were purchased at 100 µg/mL. Samples were prepared at 500 ng/mL by diluting 50 µL of each glucuronide's stock concentration in 9700 µL of UTAK urine. Each sample had 50 µL of the glucuronide solution added. The glucuronide solution was used as "unknown" in the research so that the hydrolysis efficiency could be determined.

Internal Standard Preparation

Internal standard was made that included the respective deuterated standards listed in Table 4.

Table 4. Deuterated Internal Standards. List of the drugs and their respective deuterated standards.

DRUG	DEUTERATED INTERNAL STANDARD
BUPRENORPHINE	Diazepam-D5
LORAZEPAM	Diazepam-D5
OXAZEPAM	Diazepam-D5
CODEINE	Codeine-D6
MORPHINE	Morphine-D6
THCA	THCA-D3

The internal standard mix was made by adding 50 µL of each deuterated drug to 4800 µL of UTAK urine. To each of the samples and the calibration curve, 10 µL of this solution was added. The internal standard was added so that a comparison could be made to the samples to determine how much of the drug was present in the sample.

Methods

The OSU-CLS clinically validated dilute-and-shoot method was used to prepare the calibrators and glucuronide samples for analysis. The calibration curve was treated the same for all 6 enzymes. One difference between the two curves that were used was the enzyme. In the first curve, the hydrolysis did not include the enzyme, only 40 µL of acetate buffer and 10 µL of internal standard mix was added. When the CLS curve was used, the enzyme was included in the hydrolysis solution.

The samples were run in triplicate. The hydrolysis preparation was determined by the manufacturer’s recommendations. Those recommendations, shown in Table 5, include the amount of beta-glucuronidase added, the incubation time, and the incubation temperature.

Table 5. Manufacturer's Recommendations. Recommendations from the manufacturer on how much enzyme to add, incubation time and incubation temperature.

Enzyme	Beta-Glucuronidase (µL)	Incubation Time	Incubation Temperature (°C)
Company A	10	2 hours	55
Company B	20	30 mins	55
Company C	5	1 hour	65
Company D	5	1 hour	60
Company E	25	2 hours	60
Company F	10	2 hours	60

There were two constant parameters: amount of internal standard present in each sample, 10 µL, and amount of urine in each sample, 50 µL. The amount of acetate buffer was determined with a final volume of 50 µL. Once the samples were incubated, sample diluent was added to dilute the sample prior to analysis on the LC-MS/MS. The sample diluent consisted of a 19:1 ratio of water to methanol. The samples were centrifuged for 10 minutes at 13,000 rpm. Afterwards, 200 µL aliquots of the sample were put into amber injection vials and loaded on the autosampler.

The LC parameters were based on OSU-CLS's method. Those parameters are located in Table 6.

Table 6. LC-MS/MS Instrument Parameters. These are the parameters for the LC-MS/MS.

LC Parameters for Shimadzu	
Flow Rate	0.35 mL/min
Column Oven Temperature	30°C
Injection Volume	10 µL
MS Parameters for Shimadzu	
Probe Position	+1.5 mm
DL Temperature	250°C
Heat Block Temperature	400°C
Nebulizing Gas Flow	3 L/min
Drying Gas Flow	20.0 L/min
CID Gas	230 kPa
Interface Voltage	4.5 kV
Detector Voltage	1.74 kV

Two mobile phases were used with the method: mobile phase A and mobile phase B. Mobile phase A consisted of 2 mM ammonium formate and 0.1% formic acid in water. Mobile phase B consisted of 2 mM ammonium formate and 0.1% formic acid in methanol. The gradient of the mobile phase was as follows: concentration of mobile phase B holds steady at 10% until 1 minute and 40 seconds where the concentration increases to 35%. Concentration holds at that point until 2 minutes and 54 seconds when the concentration increases to 100%. Concentration holds at 100% for a minute where concentration starts to decrease back down to 10%. A 50:25:25 mix of methanol to isopropyl alcohol to water was used as the Needle Rinse.

Data Analysis

Data analysis was performed in Browser in the Shimadzu LabSolutions software program. From the data, Microsoft Excel was used to calculate a hydrolysis efficiency for each beta-glucuronidase enzyme. The hydrolysis efficiency was calculated using the concentration outputted from LabSolutions. Once the hydrolysis efficiencies for each beta-glucuronidase enzyme was determined, the results were used in conjunction with specific guidelines to determine which beta-glucuronidase enzyme performed the best and statistical tests were performed using Microsoft Excel and GraphPad Prism.

CHAPTER IV

RESULTS

Hydrolysis Efficiency

Hydrolysis efficiencies were calculated using the equation found in Figure 2 where concentration was the amount of drug that was detected in the urine sample.

$$\frac{concentration\ (\frac{ng}{mL})}{500\ ng/mL} X\ 100 = hydrolysis\ efficiency$$

Figure 2: *Hydrolysis Efficiency Equation*. Equation used to calculate the hydrolysis efficiency of each enzyme and drug

Once the hydrolysis efficiency was calculated for all three samples, an average was found. Those averages are shown in Table 7.

Hydrolysis Efficiency (%)						
Enzyme	**Company A**	**Company B**	**Company C**	**Company D**	**Company E**	**Company F**
Buprenorphine	70.3	76.8	72.4	68.4	69.3	73.6
	65.3	62.3	77.2	75.3	63.6	64.5
	63.9	60.4	70.7	73.2	75.3	62.8
Codeine	22.3	49.1	49.8	22.1	8.7	59.3
	20.2	52.3	45.9	20.6	8.9	56.7
	19.3	47.7	49.6	21.0	8.8	49.1
Lorazepam	60.7	58.3	97.3	90.0	97.4	87.5
	56.6	53.6	95.0	98.0	88.3	84.9
	51.9	49.7	104.4	102.1	94.9	84.6
Morphine	50.6	61.7	60.9	64.2	57.7	62.7
	57.6	56.8	63.5	63.3	53.8	62.7
	50.4	50.2	65.3	65.9	57.9	59.3
Oxazepam	73.3	75.0	68.0	60.3	74.5	65.6
	69.6	64.4	64.7	69.1	61.2	62.5
	63.0	61.0	70.1	61.3	73.2	58.1
THCA	28.0	96.5	42.5	75.1	78.9	72.0
	28.3	92.4	51.7	72.1	79.1	78.2
	24.3	85.6	50.8	63.4	79.0	67.8

Table 7: *Hydrolysis Efficiencies.* Shown are the hydrolysis efficiencies for companies and the individual drugs.

The notable values to mention are from lorazepam, which shows a hydrolysis efficiency of higher than 100% for companies C and D. In these particular samples, extra lorazepam was not made, the efficiency was higher due to instrument variability.

Statistical Analysis

Analysis of variance (ANOVA) methods were used to analyze each individual drug. In total, 6 ANOVAs were performed. If the ANOVA was significant, a multiple comparisons post-test was performed to determine the significance between each enzyme with the drug classes. The ANOVA results for each individual drug class are found in Tables 8-13. A significance level of 0.05 was used.

ANOVA - Buprenorphine

Source of Variation	SS	df	MS	F	P-value
Groups	3568.144478	5	713.6288956	0.935988	0.49193
Error	9149.204724	12	762.433727		
Total	12717.3492	17			

Table 8: *ANOVA for Buprenorphine*. Results for the ANOVA for Buprenorphine

ANOVA - Codeine

Source of Variation	SS	df	MS	F	P-value
Groups	140585.0803	5	28117.01605	162.5933	1.36E-10
Error	2075.141628	12	172.928469		
Total	142660.2219	17			

Table 9: *ANOVA for Codeine*. Results for the ANOVA for Codeine.

ANOVA - Lorazepam

Source of Variation	SS	df	MS	F	P-value
Groups	156492.1188	5	31298.42376	60.61009	4.26E-08
Error	6196.676039	12	516.3896699		
Total	162688.7948	17			

Table 10: *ANOVA for Lorazepam.* Results for the ANOVA for Lorazepam.

ANOVA - Morphine

Source of Variation	SS	df	MS	F	P-value
Groups	7920.793641	5	1584.158728	5.722944	0.0063
Error	3321.700489	12	276.8083741		
Total	11242.49413	17			

Table 11: *ANOVA for Morphine.* Results for the ANOVA for Morphine.

ANOVA - Oxazepam

Source of Variation	SS	df	MS	F	P-value
Groups	3268.403014	5	653.6806028	0.875598	0.525682
Error	8958.639679	12	746.5533066		
Total	12227.04269	17			

Table 12: *ANOVA for Oxazepam.* Results for the ANOVA for Oxazepam.

ANOVA - THCA

Source of Variation	SS	df	MS	F	P-value
Groups	203616.4669	5	40723.29339	77.98325	9.99E-09
Error	6266.468047	12	522.2056706		
Total	209882.935	17			

Table 13: *ANOVA for THCA.* Results for the ANOVA for THCA.

Cost Efficiency

Cost efficiency was calculated for each individual enzyme. Calculations were done to determine the cost per sample, personnel cost and the total cost of the enzyme per sample. In order to calculate the cost per sample, the amount of samples per bottle had to calculated. The amount of samples per bottle was dependent on how much of the enzyme was needed for each sample and how big was the bottle of enzyme. Those numbers are found in Table 14.

Enzyme	Cost Per Bottle ($)	Amt in Bottle (µL)	Amt of Enzyme Per Sample (µL)	Amt of Samples Per Bottle	Cost Per Sample
Company A	247.50	20,000	10	2,000	$0.12
Company B	80.00	5,000	20	250	$0.32
Company C	410.00	10,000	5	5,000	$0.08
Company D	138.00	10,000	5	2,000	$0.07
Company E	200.00	10,000	25	400	$0.50
Company F	80.00	5,000	10	500	$0.16

Table 14: *Enzyme Amounts in Bottle*. Amount of enzyme in each companies bottle as well as how many samples can be completed using just one bottle. The cost per sample was determined using cost per bottle and amount of samples in bottle.

After determining how many sample are in each bottle, the cost of enzyme per sample was calculated.

Personnel cost was another component to consider when thinking of how to help laboratories out financially. By taking a base rate of $20/hour for personnel, the cost of personnel time could be multiplied by the incubation time to determine how much it would cost the laboratory to run one sample. The personnel cost is shown in Table 15.

Enzyme	Incubation Time (min)	Total Personnel Cost
Company A	120	$40
Company B	30	$10
Company C	60	$20
Company D	60	$20
Company E	120	$40
Company F	120	$40

Table 15: *Total Personnel Cost*. The personnel cost which was calculated using the incubation time and hourly rate of $20/hour.

After determining the personnel cost, the total cost of a sample was calculated. The total cost of a sample is the cost per sample plus the personnel cost. The total cost for each enzyme is found in Table 16.

Enzyme	Cost Per Sample	Personnel Cost	Total Cost
Company A	$0.12	$40	$40.12
Company B	$0.32	$10	$10.32
Company C	$0.08	$20	$20.08
Company D	$0.07	$20	$20.07
Company E	$0.50	$40	$40.50
Company F	$0.16	$40	$40.16

Table 16: *Total Cost Per Sample*. Total cost per sample is the cost per sample plus the personnel cost.

When determining which of the enzymes was the best overall, guidelines were set in place to help determine the best overall enzyme prior to any statistical test. It was desired to have the top enzyme have specific enzyme performance variables. The guidelines used were the enzyme must have a conversion rate of no less than 40% and work well across the different drug classes. It must also have a low total cost which includes the personnel cost and cost per sample so that it can be cost efficient for a laboratory.

The first guideline was that the enzyme must have a conversion rate of no less than 40%. Companies A, D, & E were all eliminated because they had at least one drug that was below the 40% cutoff. There were no companies that were eliminated due to not working well across the drug classes. Companies B, C & F were the last three companies left. An ANOVA was completed as well as the multiple comparisons to determine if there was any significant difference between these three companies. Once the statistical tests were complete, it was found that there was no significant difference between 4 of the 6 drugs. Lorazepam and THCA were the only analytes that had any significant differences. Between the two drugs, THCA is the hardest one to detect for the clinical laboratory method used in this research. By looking at the descriptive statistics, lorazepam was significantly better for Company C and THCA was significantly better for Company B. For THCA, Company B had a 91% hydrolysis efficiency compared to 48% for Company C. If a laboratory was looking for benzodiazepines specifically then Company C would be the obvious choice. However, in this research, an enzyme was sought that would work well across multiple drug classes, including THCA, and in this case, Company B was the clear best enzyme. The last guideline is that one that helped narrow down the top company. When looking at Table 18, one would assume that Company C would be the obvious choice because the cost per sample is lower. However, after looking at Table 16, the choice becomes clear that the top company would be Company B because Company C's enzyme cost almost four times the amount of Company B. Also, Company B's personnel cost is half of what Company C's personnel cost is. Therefore, Company B's enzyme is the best overall enzyme due to good hydrolysis efficiency and low overall personnel cost.

CHAPTER V

DISCUSSION

Beta-glucuronidases are a critical component of drug testing as they are a vital component of the dilute and shoot process by converting the drug to its free form so that it can be easily detected by the LC/MS/MS. The enzymes come in various different forms such as *patella vulgata*, *red abalone, abalone* and *recombinant* organisms. Each of the different enzymes can work well for certain drug classes, and previous research has investigated which enzymes work well for each drug class. When a larger pain panel that contains multiple drug classes is used clinically, ideally, it will use an enzyme that will work well across all the classes. This increases the amount of the different drug classes such, as benzodiazepines, opioids, and cannabinoids available for detection and helps avoid false negatives.

There were different factors in determining which enzyme was the best overall, including efficiency and cost. The variables of significance were incubation time, incubation temperature and amount of enzyme added. Incubation time and amount of enzyme are the two biggest factors to consider. In regard to incubation time, some enzymes have to incubate for 2 hours and some for just 30 minutes. If a 2-hour enzyme can produce the same results as one that incubates for 30 minutes, then results can be produced faster because samples don't have to be incubated as long. Another factor to consider is the amount of enzyme that goes into the sample. The variation of the size of the bottle and how much enzyme is used per sample can not only help the laboratory with the wet lab work but it can also help a laboratory financially. The actual cost per specimen analyzed needs to be considered when determining the best overall enzyme. The size bottle that a laboratory needs will depend on how many samples a laboratory is running per day. If a laboratory is running over 100 samples a day, then they need an enzyme that will be cost-efficient for the laboratory in regards to how many samples are analyzed. These factors are what led to the decision that Company B was the best overall enzyme in this research.

In regard to the guideline of working well across the different drugs classes, it was found that while some enzymes worked well across the different drugs classes, they didn’t necessarily work well within the drug class. Most research that has been done looking at the hydrolysis efficiency with beta-glucuronidase enzymes will look at one drug in a drug class, such as morphine-3-glucuronide. For example, researchers are looking at benzodiazepines and decide to use lorazepam glucuronide and use Company C’s enzyme. As shown in this research, Company C’s enzyme has a 98.9% hydrolysis efficiency for lorazepam glucuronide. Most researchers would stop there and conclude that enzyme C works well for benzodiazepines, but looking at the hydrolysis efficiency of 67.6% for oxazepam for Company C, it is apparent that the excellent performance on one benzodiazepine doesn’t ensure high quality with all benzodiazepines.

Another factor to also consider when determining which enzyme is the best is the cleanliness of the enzyme. If the enzyme is dirty and has unnecessary particles in it, then it may cause interferences and clogging in the LC system. This plays a factor because an enzyme could be 80% efficient but because of how dirty the enzyme is and the interferences it will cause; the enzyme might appear to only be 60% efficient. The cleanliness of the enzyme is important in determining if the enzyme will have a good hydrolysis efficiency.

Another consideration that came to light after determining our top enzyme was the laboratories' batch size. It was determined that if a laboratory was to have a batch size of 50 samples or more, the cost savings from Company B would start to diminish. If the laboratory's batch size is over 50 samples and they are not concerned with their sensitivity to THCA, Company C would be the route to go. However, if a laboratory is concerned with their sensitivity to THCA, Company B would be the best choice even though the cost savings of Company B would diminish above 50 samples.

After this work was completed, each company's technical notes were reviewed to see if this study's hydrolysis efficiencies were consistent with their expected. For most of the companies, the hydrolysis efficiencies observed during this research were lower than what the company indicated it would be. One of the companies didn't have any published research on what the hydrolysis efficiency should be for certain classes, so there was no point of comparison.

There is also a chance that an enzyme that is used could create a false negative result depending on which company's enzyme a laboratory is using. If a particular company's enzyme has a low hydrolysis efficiency for a particular drug, then it will only hydrolyze a portion of the glucuronides present which could lead to the drug being below the limit of quantitation for the laboratory which would result in a negative result, when the drug is in fact present. If that sample was then sent to another laboratory who uses an enzyme that has a great hydrolysis for that same drug, then it could be above their lower limit of quantitation which would result in a true positive result. Having an enzyme that has a good hydrolysis efficiency will reduce the chances of a false negative.

In terms of future research, it might be beneficial to attempt to optimize conditions for Company B's enzyme further and investigate making it better. It is possible to increase or decrease the incubation temperature, times and amount of enzyme to determine if the same results are achieved as the current study. Other glucuronides of interest to the clinical laboratory could also be used with the same method in this research to see if there are any variations between the drug classes. Lastly, this research used an indirect measure of glucuronide hydrolysis efficiency by measuring formation of the target drugs. Future research may involve direct measurement of glucuronides by LC-MS/MS to confirm that any target analyte created is due to loss of glucuronide.

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